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(54) Title: MUTANT CYTOKINES HAVING INCREASED RECEPTOR AFFINITY (57) Abstract A variant of a naturally-occurring cytokine having a Phe-Leu or Tyr-Leu sequence in a helical domain and a negatively charged amino acid within two amino acids immediately upstream or downstream from said Phe-Leu or Tyr-Leu sequence, said variant having an increased affinity for the receptor of said naturally-occurring cytokine by virtue of a neutral or basic amino acid having been substituted for a said negatively charged amino acid.		

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MUTANT CYTOKINES HAVING INCREASED RECEPTOR AFFINITY

Background of the Invention

The invention relates to mutant cytokines having
5 an altered receptor affinity.

PCT Application PCT/US86/02464 discloses native
and mutant forms of interleukin-4 from a variety of
mammalian sources.

Taniguchi et al. (U.S. Patent No. 4,738,927)
10 discloses a gene encoding interleukin-2.

Murphy et al. (U.S. Patent No. 4,675,392) disclose
cytotoxic fusion proteins that include a portion of
interleukin-2.

Bazan (*Immunology Today* 11:350, 1990) describes
15 structural models of cytokine receptor interactions.

Cunningham et al., (*Science* 244:1081, 1989)
describe alanine substitution mutants of human growth
hormone, including a substitution at Glu¹⁷⁴ which
increases affinity.

20 Summary of the Invention

The invention features variants of naturally-
occurring cytokines containing a Phe-Leu or Tyr-Leu
sequence in an alpha helical domain and a negatively-
charged amino acid (Glu or Asp) within two amino acids
25 immediately upstream or downstream from the Phe-Leu or
Tyr-Leu sequence; the variants have an increased affinity
for the receptor of the naturally-occurring cytokine by
virtue of a neutral (Gly, Ala, Val, Ile, Leu, Phe, Pro,
Met, Ser, Thr, Tyr, Trp, Asn, Gln, Lys) amino acid which
30 is substituted for the negatively-charged amino acid
which is within two amino acids immediately upstream or
downstream of the Phe-Leu or Tyr-Leu sequence.

In general, the invention features a variant of a
naturally-occurring cytokine having a Phe-Leu or Tyr-Leu

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sequence in a alpha helical domain and a negatively charged amino acid within two amino acids immediately upstream or downstream from the Phe-Leu or Tyr-Leu sequence, the variant having an increased affinity for
5 the receptor of the naturally-occurring cytokine by virtue of a neutral amino acid having been substituted for the negatively charged amino acid. Receptor affinity can be measured using standard receptor binding assays.

In preferred embodiments, the negatively charged
10 amino acid is aspartic acid or glutamic acid; and the naturally occurring cytokine is an interleukin. In a more preferred embodiment, the interleukin is IL-4. In a even more preferred embodiment, the IL-4 is human IL-4.

In a related aspect, the invention features a
15 hybrid molecule which includes a first and a second portion joined together covalently, the first portion includes a receptor-binding portion of the above-described variant cytokine and the second portion includes a molecule having enzymatic activity. In a
20 preferred embodiment, the enzymatic activity is capable of decreasing cell viability. By "reduces viability" is meant kills or inhibits proliferation.

In a preferred embodiment, the second portion includes a cytotoxin. In a more preferred embodiment,
25 the cytotoxin is a fragment of a peptide toxin which is enzymatically active but which does not possess generalized eukaryotic receptor binding activity. In an even more preferred embodiment, the fragment of a peptide toxin includes fragment A of diphtheria toxin and enough
30 of fragment B of diphtheria toxin to form a pore in a cell membrane.

The variants of the invention generally are based on cytokines which, like human IL-4, have the sequence Phe-Leu or Tyr-Leu in an alpha helix; the alpha helix
35 generally is within the carboxy half of the cytokine and

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is usually the most carboxy-terminal alpha helix. Cytokines having the aforementioned characteristics are referred to as human IL-4 related cytokines. Examples of human IL-4 related cytokines include: human prolactin, 5 human interleukin-2, human interleukin-6, and human interleukin-8. Generally, the alpha helices of human IL-4 related cytokines encompassing the above-described Phe-Leu (or Tyr-Leu) sequence are amphiphilic. With regard to protein structure an alpha helix is a structure in 10 which the backbone of the peptide chain forms a helix. In such a helix there are roughly 3.6 amino acids per turn of the helix. An amphiphilic alpha helix has a charged face and an uncharged face.

A cytokine has characteristics which permit its 15 classification as a human IL-4 related cytokine if two criteria are met. First, there must be an Phe-Leu (or Tyr-Leu) sequence in the carboxy terminal half of the cytokine. Second, the Phe-Leu or Tyr-Leu sequence must be within an alpha helix. While extensive structural 20 analysis, e.g., x-ray crystallography or NMR analysis, may be required to make such a determination with complete certainty, for the purposes of the identifying human IL-4 related cytokines a determination that the Phe-Leu or Tyr-Leu sequence lies within a region which 25 has the characteristics of an alpha helix forming sequence is sufficient. Such secondary structure predictions can be made using well-known techniques (Chothia et al., *J. Biol. Chem.* 145:215, 1981; Cohen et al., *J. Mol. Biol.* 132:275, 1979; Cohen et al., *Proteins* 30 7:1, 1990; Cornette et al., *J. Mol. Biol.* 195:659, 1987; Bazan et al., *Proc. Natl. Acad. Sci. USA* 85:7872, 1988; Richardson et al., *Science* 240:1648, 1988). Secondary structure prediction can also be made using a computer program such as PCGENE™ (IntelliGenetics, Mountain View, 35 CA). If such analysis indicates that the Phe-Leu (or

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- Tyr-Leu) sequence lies within an alpha helix, then the cytokine is a human IL-4 related cytokine. In general, the alpha helix containing the Phe-Leu or Tyr-Leu sequence is the most carboxy-terminal alpha helix. The finding that the alpha helix is amphipathic gives added assurance that the cytokine is a human IL-4 related cytokine, but is not required to fulfill the criteria for inclusion. Bazan (*supra*) describes structural features of cytokines.
- 10 The increased affinity cytokine variants of the invention can be used in any application currently employing the naturally occurring cytokine. Of particular importance are hybrid toxin molecules in which the cytokine or a receptor binding portion thereof
- 15 replaces the generalized eukaryotic binding domain of toxins such as diphtheria toxin to form a hybrid molecule capable of selectively targeting an unwanted class of cells, e.g., IL-2 receptor- or IL-4 receptor-bearing cells involved in human disease such as
- 20 T-cell lymphoma. The increased affinity of the variants of the invention for the receptor will render the hybrid molecules more toxic than hybrids made using the corresponding naturally-occurring cytokine, and thus smaller dosages will be required to achieve the same
- 25 therapeutic effect.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description.

- 30 The drawings are first briefly described.

Fig. 1 is a graph illustrating the effect of various molecules on binding of [125 I]-mIL-4 bound to P815 cells. The percentage of the maximum amount [125 I]-mIL-4 bound is plotted as a function of the concentration (M)

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of mIL-4 (O), DAB₃₈₉-mIL-4(D⁴⁹⁵ →N) (Δ), DAB₃₈₉-mIL-4 (O), DAB₃₈₉-mIL-4(F⁴⁹⁶ →A) (♦), or DAB₃₈₉-mIL-4(F⁴⁹⁶ →P) (■).

Fig. 2 is a graph illustrating the effect of DAB₃₈₉-mIL-4 and DAB₃₈₉-mIL-4 variants on incorporation of [14C]-leucine into P815 murine mastocytoma cell. Percent incorporation (relative to untreated cells) is plotted as a function of the concentration (M) of DAB₃₈₉-mIL-4 (O), DAB₃₈₉-mIL-4(D⁴⁹⁵ →N) (•), DAB₃₈₉-mIL-4(F⁴⁹⁶ →A) (♦), or DAB₃₈₉-mIL-4(F⁴⁹⁶ →P) (■).

10 Cytokine Structure and Receptor Binding

A number of cytokines have similar structural elements. In particular, several that are known to bind to one or another member of the hematopoietin cytokine receptor superfamily are predicted to have a carboxy terminal alpha helix. In many cases the alpha helix is substantially amphiphilic in nature. Importantly, each has a highly conserved Phe-Leu (or Tyr-Leu) sequence within the amphipathic helix. Murine and human interleukin-4 are examples of cytokines having the above-described conserved structure.

Described below are a series of experiments employing DAB₃₈₉-mIL-4, a murine interleukin-4 diphtheria toxin-related fusion protein. These experiments demonstrate the importance of certain structural elements to receptor binding. DAB₃₈₉-mIL-4 is created by replacing the generalized cell binding domain (deletion of 97 amino acids Thr³⁸⁷ to His⁴⁸⁵ of diphtheria toxin with murine interleukin-4 (mIL-4). Thus, the amino terminus of mIL-4 is linked by a peptide bond to a carboxy-terminal portion of diphtheria toxin. This molecule is selectively toxic to cells bearing the mIL-4 receptor. Using site directed and in-frame deletion mutagenesis to alter the mIL-4 portion of DAB₃₈₉-mIL-4, we have found that deletion of the carboxy-terminal 15 amino acids of mIL-4, substitution of Phe⁴⁹⁶ with either Pro, Ala, or Tyr, or

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substitution of Leu⁴⁹⁷ with either Ala or Glu decreases binding to the mIL-4 receptor and cytotoxicity. In contrast, the substitution of the negatively charged residue Asp⁴⁹⁵ with Asn results in a four-fold increase in cytotoxic potency and binding affinity to mIL-4 receptor bearing cells *in vitro*.

DAB₃₈₉mIL-4 Variants

DAB₃₈₉mIL-4 is a fusion protein in which the receptor binding domain of native diphtheria toxin has been replaced by murine interleukin-4 (mIL-4). DAB₃₈₉mIL-4 selectively binds to eukaryotic cells expressing the murine IL-4 receptor (mIL-4R), causing them to internalize the DAB-derived portion of the molecule which inhibits protein synthesis in the cells, causing cell death. DAB₃₈₉mIL-4 cytotoxicity is inhibited by excess mIL-4 or antibodies directed against mIL-4, demonstrating that entry of DAB₄₈₉mIL-4 into target cells is mediated by the mIL-4 receptor.

We have modified the mIL-4 portion of DAB₃₈₉mIL-4 to create variants with altered toxicity towards mIL-4R bearing cells. Since the change is in the mIL-4 portion of the molecule, it follows that altered toxicity is caused by altered affinity of the molecule for the mIL-4 receptor. Thus, these same mutations, when introduced into a related fusion protein, should alter its affinity for its receptor in an analogous fashion.

Described below are several DAB₃₈₉mIL-4 variants created by site directed mutagenesis. The ability of each variant to bind and intoxicate cells bearing the mIL-4 receptor was tested. The results permit prediction of the effect of mutation on IL-4 related cytokines including human IL-2, human IL-6, human IL-8.

Several in-frame deletions and point mutations were introduced into the mIL-4 segment of the structural gene encoding DAB₃₈₉mIL-4 carried on plasmid pFL389

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(Lakkis et al., *Eur. J. Immunol.* 21:2253, 1991). Briefly, oligonucleotide site-directed mutagenesis was performed using a T7-Gen in vitro mutagenesis kit (United States Biochemical, Cleveland, OH) based on the previously published work (Vandeyar et al., *Gene* 65:129, 1988). The *mIL-4* cDNA obtained from plasmid pFL389 was cloned into the *SphI*-*HindIII* sites of M13(mp19) viral vector. The single stranded viral DNA served as the template in all mutagenesis reactions. Oligonucleotide primers were synthesized on an Applied Biosystems 391A DNA synthesizer. Correct base substitution was confirmed by DNA sequencing (Sanger et al., 1977). The *mIL-4* cDNA fused to a truncated diphtheria toxin gene on the expression plasmid pFL389 was then substituted with the respective mutated *mIL-4* cDNAs. In-frame deletion mutations were constructed by utilizing convenient restriction endonuclease sites in the fusion gene and by using oligonucleotide linkers. *E. Coli* JM101 (BRL/GIBCO, Bethesda, MD) was used throughout.

Recombinant DAB₃₈₉*mIL-4* was prepared from recombinant *E. coli* grown in 10 liters of M9 minimal medium (1mM MgSO₄, 0.1mM CaCl₂, 0.0005% thiamine, 0.5% glycerol) supplemented with 1% Casamino acids (Difco) and 100 µg/ml ampicillin in a New Brunswick Microferm.

Cultures were incubated at 30°C and sprayed with air at 10 L/min. When the absorbance (A_{590nm}) of the culture reached 0.3 - 0.5, expression of the chimeric *tox* gene was induced by the addition of 1 gm isopropyl-β-D-thiogalactopyranoside (IPTG) (United States Biochemicals, Cleveland, OH). Ninety min after induction, bacteria were harvested by centrifugation, resuspended in lysis buffer (50mM KH₂PO₄, 10mM EDTA, 750mM NaCl, 0.1% Tween 20, pH 8.0) and disrupted by sonication (Branson). The bacterial lysate was centrifuged at 2,500 x g for 20 min at 4°C to remove whole bacteria and debris, and the

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clarified lysate was applied to an anti-diphtheria toxin immunoaffinity column. Bound proteins were eluted from the immunoaffinity column with elution buffer (4M guanidine hydrochloride, 100mM KH_2PO_4 , 0.1% Tween 20, pH 7.2). Recombinant DAB₃₈₉-mIL-4 fusion toxins were further purified by high pressure liquid chromatography on a 7.5 x 600mm G4000PW column (TosoHass). Prior to use the fusion toxins were exhaustively dialyzed against phosphate buffered saline (PBS, pH 7.4). Protein concentration was determined by the Bradford method (Pierce Chemical Co., Rockford, IL).

Table 1 summarizes the single amino acid substitutions introduced into DAB₃₈₉mIL-4. In addition, two deletion mutants were expressed and purified:

15 DAB₃₈₉-mIL-4(Δ 495-509) is a variant of DAB₃₈₉-mIL-4 that lacks the 15 carboxy-terminal amino acids, while DAB₃₈₉-mIL-4(Δ 390-475) consists of the first 389 amino acids of diphtheria toxin fused directly to the fifth cysteine residue in mIL-4 by a His-Ala encoding

20 oligonucleotide linker. Analysis of DAB₃₈₉-mIL-4 and mutant fusion toxins on coomassie blue stained 12% SDS-polyacrylamide gels indicated that the proteins were highly purified. Their electrophoretic mobilities corresponded to their respective molecular weights as

25 deduced from DNA sequence analysis. However, DAB₃₈₉-mIL-4(F⁴⁹⁶→P), in which Phe⁴⁹⁶ is replaced with a Pro residue, had slightly aberrant electrophoretic mobility.

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TABLE 1: DAB₃₈₉ mIL-4 Variants

PLASMID	TOX GENE PRODUCT
5 pFL389	DAB ₃₈₉ -mIL-4
pFL389 (15)	DAB ₃₈₉ -mIL-4 (Δ495-509)
pFL389 (86)	DAB ₃₈₉ -mIL-4 (Δ390-475)
pFL389 (F→P)	DAB ₃₈₉ -mIL-4 (F ⁴⁹⁶ →P)
pFL389 (F→A)	DAB ₃₈₉ -mIL-4 (F ⁴⁹⁶ →A)
10 pFL389 (F→Y)	DAB ₃₈₉ -mIL-4 (F ⁴⁹⁶ →Y)
pFL389 (L→A)	DAB ₃₈₉ -mIL-4 (L ⁴⁹⁷ →A)
pFL389 (L→E)	DAB ₃₈₉ -mIL-4 (L ⁴⁹⁷ →E)
pFL389 (D→N)	DAB ₃₈₉ -mIL-4 (D ⁴⁹⁵ →N)
pFL389 (K→A)	DAB ₃₈₉ -mIL-4 (K ⁴⁹⁴ →A)
15 pFL389 (K→L)	DAB ₃₈₉ -mIL-4 (K ⁴⁸⁹ →L)
pFL389 (C→G)	DAB ₃₈₉ -mIL-4 (C ⁴⁷⁶ →G)

In vitro cytotoxic activity of DAB₃₈₉-mIL-4 and related variants on mIL-4 receptor bearing cells

The cytotoxic action of DAB₃₈₉-mIL-4 requires binding to the mIL-4 receptor, receptor-mediated endocytosis, passage through an acidic compartment, and delivery of the ADP-ribosyltransferase component of the fusion toxin into the cytosol (Lakkis et al., *supra*). In order to investigate the effect of mutations in the mIL-4 component of DAB₃₈₉-mIL-4 on the function of this fusion protein, we have tested the *in vitro* cytotoxic potency of DAB₃₈₉-mIL-4 and related variant fusion toxins on the P815 murine mastocytoma cell line by means of a [¹⁴C]-leucine uptake assay. Briefly, P815 murine mastocytoma cells (American Type Culture Collection, Rockville, MD), maintained in RPMI 1640 medium supplemented with 10%

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fetal bovine serum (Cellec, BRL/GIBCO, Bethesda, MD), 2 mM glutamine, and penicillin and streptomycin to 50 IU and 50 μ g/ml, respectively. For cytotoxicity assays, cells were seeded in 96-well plates (Linbro-Flow laboratories, McLean, VA) at a concentration of 1×10^4 per well. Fusion toxins were added in varying concentrations and the cultures were incubated for 40 hours at 37°C in a 5% CO₂ incubator. Following incubation, the cells were pulsed with [¹⁴C]-leucine and radioactivity incorporated into proteins was measured essentially as described earlier by Williams et al. (*J. Biol. Chem.* 265:11885, 1990). All assays were performed in quadruplicate. Dose response curves (Fig. 1) compare the percent incorporation of [¹⁴C]-leucine by the fusion toxin treated cultures relative to untreated controls.

The results of the cytotoxicity assays are presented in Table 2, where IC₅₀ is the concentration of fusion toxin required for a 50% reduction in protein synthesis. Values are means of 3-5 independent experiments. Standard deviations were within 25% of the respective means.

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TABLE 2: Cytotoxicity and receptor binding affinity of DAB₃₈₉-mIL-4 and variants.

	Fusion Toxin	IC ₅₀ (M)	K _i (M)
5	DAB ₃₈₉ -mIL-4	1 x 10 ⁻⁹	2.1 x 10 ⁻⁷
	DAB ₃₈₉ -mIL-4 (Δ495-509)	> 1 x 10 ⁻⁷	> 1.0 x 10 ⁻⁶
	DAB ₃₈₉ -mIL-4 (Δ390-475)	> 1 x 10 ⁻⁷	> 1.0 x 10 ⁻⁶
	DAB ₃₈₉ -mIL-4 (F ⁴⁹⁶ →P)	> 1 x 10 ⁻⁷	> 1.0 x 10 ⁻⁶
10	DAB ₃₈₉ -mIL-4 (F ⁴⁹⁶ →A)	5 x 10 ⁻⁸	> 1.0 x 10 ⁻⁶
	DAB ₃₈₉ -mIL-4 (F ⁴⁹⁶ →Y)	3 x 10 ⁻⁸	> 1.0 x 10 ⁻⁶
	DAB ₃₈₉ -mIL-4 (L ⁴⁹⁷ →A)	3 x 10 ⁻⁸	> 1.0 x 10 ⁻⁶
	DAB ₃₈₉ -mIL-4 (L ⁴⁹⁷ →E)	> 1 x 10 ⁻⁷	> 1.0 x 10 ⁻⁶
	DAB ₃₈₉ -mIL-4 (D ⁴⁹⁵ →N)	4 x 10 ⁻¹⁰	5.2 x 10 ⁻⁸
15	DAB ₃₈₉ -mIL-4 (K ⁴⁹⁴ →A)	1 x 10 ⁻⁹	4.0 x 10 ⁻⁷
	DAB ₃₈₉ -mIL-4 (K ⁴⁸⁹ →L)	2 x 10 ⁻⁹	3.8 x 10 ⁻⁷
	DAB ₃₈₉ -mIL-4 (C ⁴⁷⁶ →G)	> 1 x 10 ⁻⁷	> 1.0 x 10 ⁻⁶

Deletion of the carboxy-terminal 15 amino acids of DAB₃₈₉-mIL-4 results in a complete loss of cytotoxic activity in the mutant DAB₃₈₉-mIL-4(Δ495-509) (Table 1). This deletion encompasses residues Asp⁴⁹⁵-Met⁵⁰⁴ and indicates that the C-terminal region of IL-4 is required for the cytotoxic activity of the fusion toxin. In order to examine whether this segment alone was capable of targeting a diphtheria toxin-based fusion protein into the cytosol of mIL-4 receptor expressing cells, we then constructed a deletion mutant of DAB₃₈₉-mIL-4 in which Thr³⁸⁹ was fused to Cys⁴⁷⁶ through an oligonucleotide linker. As shown in Table 1, DAB₃₈₉-mIL-4(Δ390-475) is also devoid of cytotoxic activity. These observations

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suggest that other regions of the mIL-4 component of DAB₃₈₉-mIL-4, acting alone or in concert, are required for biological activity of the fusion toxin.

We next examined two DAB₃₈₉mIL-4 mutants in which Phe⁴⁹⁶ is replaced with either Pro or Ala. Fig. 1, in which percent incorporation of [¹⁴C]-leucine by fusion toxin treated cultures relative to untreated controls is plotted as a function of fusion toxin concentration (M), shows that both mutations DAB₃₈₉-mIL-4(F⁴⁹⁶→P) (filled squares) and DAB₃₈₉mIL-4(F⁴⁹⁶→A) (filled diamonds) result in a marked reduction in cytotoxic potency to P815 cells. To further analyze the role of Phe⁴⁹⁶ in the cytotoxic process, the activity of DAB₃₈₉-mIL-4(F⁴⁹⁶→Y), in which Tyr was substituted for Phe⁴⁹⁶, was tested. Since tyrosine has a hydroxyl group on carbon-4 in the phenyl ring, position 496 in DAB₃₈₉-mIL-4(F⁴⁹⁶→Y) is occupied by a more polar residue which is otherwise structurally similar to phenylalanine. As shown in Table 2, this mutation, DAB₃₈₉-mIL-4(F⁴⁹⁶→Y), causes a 13-fold decrease in cytotoxic potency.

Since a Phe-Leu pair is almost invariably found in the carboxy terminal helices of human IL-4-related cytokines, we have also examined the role of Leu⁴⁹⁷ in the cytotoxic activity of DAB₃₈₉-mIL-4. Substitution of Leu⁴⁹⁷ with either an Ala or Glu [DAB₃₈₉-mIL-4(L⁴⁹⁷→A) and DAB₃₈₉-mIL-4(L⁴⁹⁷→E), respectively] results in a marked loss of cytotoxic potency (Table 2).

In contrast to the negative effect of the above substitutions on the biological activity of DAB₃₈₉-mIL-4, we have found that mutations involving some of the hydrophilic residues in the region surrounding the conserved Phe-Leu pair result in either no change or an increase in cytotoxicity. For example, substitution of Lys⁴⁹⁴ with Ala does not alter the cytotoxic potency of the resulting fusion toxin (Table 2). DAB₃₈₉-mIL-

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4(D⁴⁹⁵→N⁴⁹⁵), in which Asp⁴⁹⁵ is replaced with an uncharged Asn, is approximately four-fold more cytotoxic than the parental fusion toxin, DAB₃₈₉-mIL-4 (Table 2). In addition, substitution of Asp⁴⁹⁵ with an Ala also results in four-fold increase in cytotoxic potency. It is of interest to note that Asp⁴⁹⁵ is adjacent to the Phe-Leu pair. Mutations which increase the relative hydrophobicity of this region of the helix have resulted in an increase in the biological activity of the fusion toxin.

Although predicted carboxy-terminal alpha helix on mIL-4 displays significant amphiphilic character, the hydrophobic face of the helix is interrupted by a charged residue, Lys⁴⁸⁹. We, therefore, tested whether a Lys⁴⁸⁹ to a Leu mutation would enhance the amphiphilicity of this segment and possibly improve the cytotoxic potency of the fusion toxin (Table 2). Interestingly, DAB₃₈₉-mIL-4(Lys⁴⁸⁹→Leu) demonstrates 2-3 fold less cytotoxicity than the parental DAB₃₈₉-mIL-4 form of the fusion toxin (Table 2). Finally, substitution of Cys⁴⁷⁶ with a Gly residue led to total loss of cytotoxic activity.

Apparent affinities of DAB₃₈₉-mIL and related variant fusion toxins to the m-IL-4 receptor

The biological activity of DAB₃₈₉-mIL-4 has been shown to be mediated through the mIL-4 receptor on target cells since cytotoxicity could be specifically blocked with either excess mIL-4 or antibody to mIL-4 (Lakkis et al., *supra*). Since we have introduced mutations into the mIL-4 component of the fusion toxin, it is likely that the observed changes in the cytotoxicity of the DAB₃₈₉-mIL-4 variants are due to altered binding to the mIL-4 receptor. To test this hypothesis, we conducted a series of competitive displacement experiments using [¹²⁵I]-labeled recombinant mIL-4. (Recombinant murine IL-4,

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Sterling Drug Inc., Malvern, PA was radioiodinated using the Enzymobead reagent, BioRad, Richmond, GA).

Briefly, P815 cells were washed with tissue culture medium, resuspended in PBS (pH 7.4) containing 3 mg/ml BSA at 1×10^6 cells reaction tube, and incubated with 175 pM [125 I]-mIL-4 in the presence or absence of increasing concentrations of unlabeled recombinant mIL-4 (Sterling Drug Inc., Malver, PA) or DAB₃₈₉-mIL-4 fusion toxins for 25 minutes at 37°C under 5% CO₂. The reaction was overlaid on a mixture of 80% 550 fluid (Accumetric Inc., Elizabethtown, KN) and 20% mineral oil (Sigma, St. Louis, MO) and microcentrifuged for 2 minutes. The aqueous phase and pellet of each sample, representing free and bound ligand respectively, were then counted in a Beckman Gamma 5500 counter. Inhibition constants, K_i , were calculated from the Cheng-Prusoff equation (Cheng et al., *Biochem. Pharm.* 22:3099, 1973).

As shown in Fig. 2, where the percentage [125 I]-labeled recombinant mIL-4 bound is plotted as a function of fusion toxin concentration (M), there is a direct correlation between the cytotoxic potency of a given fusion toxin and its affinity to the mIL-4 receptor. While neither DAB₃₈₉-mIL-4(F⁴⁹⁶-P) (filled squares) nor DAB₃₈₉-mIL-4(F⁴⁹⁶-A) (filled diamonds) significantly displaced [125 I]-mIL-4 bound to P815 cells, DAB₃₈₉-mIL-4(D⁴⁹⁵-N) (open diamonds) appears to have a four-fold higher affinity than parental DAB₃₈₉-mIL-4 ($K_i = 5.2 \times 10^{-8}$ M versus 2.1×10^{-7} respectively). Displacement of mIL-4 (filled circles) was measured as a control. The K_i and IC₅₀ values for DAB₃₈₉-mIL-4 and each of the mutant fusion toxins are summarized in Table 2. The inhibition constants were calculated following the Cheng-Prusoff equation using 129 ± 5 pM (mean \pm SD of 3 independent experiments) for the radioligand equilibrium dissociation constant. Values are means of 3-5 independent

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experiments. Standard deviations were within 25% of the respective means. Taken together, the data presented in Table 1 demonstrates that mutations in the C-terminus which alter cytotoxic potency also modify the affinity of the respective fusion toxin for the mIL-4 receptor.

Structural characteristics of DAB₃₈₉-mIL-4 and related variant fusion toxins

In an attempt to explore the mechanism by which mutations in the carboxy-terminal segment of the mIL-4 component influence binding of the fusion toxin to the mIL-4 receptor, we have examined the electrophoretic mobilities of the parental and mutant fusion toxins under native, non-denaturing conditions. These studies demonstrated that these proteins are predominantly monomeric in non-denaturing buffer. These data imply that the loss in cytotoxic activity and binding affinity observed in DAB₃₈₉-mIL-4 variants is not due to increased aggregation of the respective fusion toxins. We then investigated the possibility that marked reduction in affinity could be due to altered tertiary structure of the mIL-4 component of the fusion toxin. Non-denatured fusion proteins were therefore transferred to nitrocellulose paper and immunoblotted (Towbin et al., *Proc. Natl. Acad. Sci. USA*, 76:4350, 1979) with a conformationally sensitive monoclonal antibody to mIL-4 (11B11, Genzyme, Boston, MA). DAB₃₈₉-mIL-4 is immunoblot positive when probed with 11B11 only following electrophoresis under non-denaturing conditions (Lakkis et al., *supra*). This analyzing demonstrated that DAB₃₈₉-mIL-4, as well as those variants with similar or higher cytotoxic activity and binding affinity, are strongly immunoreactive with 11B11. Conversely DAB₃₈₉-mIL-4(Δ 495-509), DAB₃₈₉-mIL-4(Δ 390-475), DAB₃₈₉-mIL-4(C⁴⁷⁶-G), DAB₃₈₉-mIL-4(F⁴⁹⁶-P) and DAB₃₈₉-mIL-4(L₄₉₇-E) which are non-toxic and fail to bind to the mIL-4 receptor are not recognized

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by the anti-mIL-4 monoclonal antibody. Conservative substitutions of Phe⁴⁹⁶ or Leu⁴⁹⁷, on the other hand, are immunoblot positive but with lower intensity than DAB₃₈₉-mIL-4. DAB₃₈₉-mIL-4(L₄₉₇→A), for example, is 13-fold less cytotoxic than DAB₃₈₉-mIL-4, has a markedly reduced affinity for the mIL-4 receptor yet is significantly reactive with 11B11.

Other Variant Human Growth Hormone-Related Cytokines

Human IL-4-related cytokines include human IL-6, human IL-8, and human IL-2. In each case the predicted amino acid sequence indicates the presence of an acidic (negatively charged) amino acid one or two amino acids upstream of a Phe-Leu (or Tyr-Leu) sequence within the carboxy-terminal portion of the molecule. Residues which could be changed to a neutral residue include position 126 of hIL-4 (U.S. Patent 5,017,691); 117 of hIL-6 (EPA 0 261 625); the E within the sequence RVVEKFLKRA of hIL-8 (Matsushima et al., *J. Exp. Med.* 167:1883, 1988); and 136 of hIL-2 (Taniguchi et al., *Nature* 302:305, 1987). These negatively charged residues may be replaced by any neutral residue as described above for IL-4. The affinity of each variant may then be tested using standard binding assays. Hybrid Molecules

A variant cytokine of the invention can be linked to other molecules (e.g., a molecule with enzymatic activity) to create hybrid molecules. These hybrid molecules will bind to cells bearing the relevant cytokine receptor. This provides a convenient method for targeting molecules to cytokine receptor bearing cells. Because the variant cytokines of the invention bind their receptors with higher affinity than the related naturally occurring cytokines, the hybrid molecules will be particularly useful for directing molecules cells bearing the relevant cytokine receptor.

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Hybrid cytotoxins are a useful class of hybrid molecule which can be created using the variant cytokines of the invention. Such molecules can be used to reduce the viability of cells bearing particular cytokine
5 receptors. In these molecules a cytotoxin is linked to the variant cytokine. Hybrid cytotoxins are described by Murphy et al. (U.S. Patent 4,675,392). Other hybrid cytotoxins are described by Murphy (PCT/US90/07619). Methods for constructing such cytotoxins are well known
10 (Murphy et al., U.S. Patent 4,675,392; Murphy, (PCT/US90/07619); Lakkis et al., *supra*; Williams et al., *J. Biol. Chem.* 265:20673, 1990; Williams et al., *J. Biol. Chem.* 265:11885, 1990). Toxicity can be tested using standard assays.

15 Other useful hybrid molecules are those in which a non-cytotoxic, enzymatically active molecule is linked to a variant cytokine of the invention. Such molecules, when taken up by the targeted cell, can be used to correct an enzyme deficiency or generate other molecules
20 within the cell by enzymatic activity. It is also possible to link a detectable label to the variant cytokines of the invention for the purpose of detectably labeling receptor-bearing cells.

Use

25 The variant cytokines of the invention can be used for the same therapeutic purposes of the related naturally occurring molecule. In addition, truncated variant cytokines which bind receptor but do not elicit the normal biological response can be used to inhibit
30 action of the naturally occurring cytokine. Because of their higher receptor affinities, such molecules employing the variant cytokines of the invention will generally be more potent than molecules employing the related naturally occurring cytokine.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT: The University Hospital

(ii) TITLE OF INVENTION: MUTANT CYTOKINES HAVING
INCREASED RECEPTOR AFFINITY

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:

10 (A) ADDRESSEE: Fish & Richardson

(B) STREET: 225 Franklin Street

(C) CITY: Boston

(D) STATE: Massachusetts

(E) COUNTRY: U.S.A.

15 (F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb

20 (B) COMPUTER: IBM PS/2 Model 50Z or 55SX

(C) OPERATING SYSTEM: MS-DOS (Version 5.0)

(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

25 (B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 07/870,500

30 (B) FILING DATE: April 17, 1992

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Paul T. Clark

35 (B) REGISTRATION NUMBER: 30,162

(C) REFERENCE/DOCKET NUMBER: 00563/053001

(ix) TELECOMMUNICATION INFORMATION:

40 (A) TELEPHONE: (617) 542-5070

(B) TELEFAX: (617) 542-8906

(C) TELEX: 200154

- 19 -

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 576
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTAGCATCTG TTGATAAACT TAATTGTCTC TCGTCACTGA CGCACAGAGC TATTG 55

AAC CCC CAG CTA GTT GTC ATC CTG CTC TTC TTT CTC GAA TGT ACC AGG 103
 15 Asn Pro Gln Leu Val Val Ile Leu Leu Phe Phe Leu Glu Cys Thr Arg
 1 5 10 15

AGC CAT ATC CAC GGA TGC GAC AAA AAT CAC TTG AGA GAG ATC ATC GGC 151
 20 Ser His Ile His Gly Cys Asp Lys Asn His Leu Arg Glu Ile Ile Gly
 20 25 30

ATT TTG AAC GAG GTG ACA GGA GAA GGG ACG CCA TGC ACG GAG ATG GAT 199
 Ile Leu Asn Glu Val Thr Gly Glu Gly Thr Pro Cys Thr Glu Met Asp
 35 40 45

25 GTG CCA AAC GTC CTC ACA GCA ACG AAG AAC ACC ACA GAG AGT GAG CTC 247
 Val Pro Asn Val Leu Thr Ala Thr Lys Asn Thr Thr Glu Ser Glu Leu
 50 55 60

30 GTG TGT AGG GCT TCC AAG GTG CTT CGT ATA TTT TAT TTA AAA CAT GGG 295
 Val Cys Arg Ala Ser Lys Val Leu Arg Ile Phe Tyr Leu Lys His Gly
 65 70 75 80

AAA ACT CCA TGC TTG AAG AAG AAC TCT AGT GTT CTC ATG GAG CTG CAG 343
 35 Lys Thr Pro Cys Leu Lys Lys Asn Ser Ser Val Leu Met Glu Leu Gln
 85 90 95

AGA CTC TTT CGG GCT TTT CGA TGC CTG GAT TCA TCG ATA AGC TGC ACC 391
 Arg Leu Phe Arg Ala Phe Arg Cys Leu Asp Ser Ser Ile Ser Cys Thr
 40 100 105 110

- 20 -

ATG AAT GAG TCC AAG TCC ACA TCA CTG AAA GAC TTC CTG GAA AGC CTA 439
Met Asn Glu Ser Lys Ser Thr Ser Leu Lys Asp Phe Leu Glu Ser Leu
115 120 125

5 AAG AGC ATC ATG CAA ATG GAT TAC TCG 466
Lys Ser Ile Met Gln Met Asp Tyr Ser
130 135

10 TAGTACTGAG CCACCATGCT TTAACCTATG AATTTTAAAT GGTTTATTT TTAATATTTA 526
TATATTTATA ATGATAAAA TAAATATTT GTATAATGTA ACAGAAAAAA 576

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Claims

1. A variant of a naturally-occurring cytokine having a Phe-Leu or Tyr-Leu sequence in a alpha helical domain and a negatively charged amino acid within two
5 amino acids immediately upstream or downstream from said Phe-Leu or Tyr-Leu sequence, said variant having an increased affinity for the receptor of said naturally-occurring cytokine by virtue of a neutral amino acid having been substituted for a said negatively charged
10 amino acid.
2. The variant cytokine of claim 1 wherein a said negatively charged amino acid is aspartic acid or glutamic acid.
3. A hybrid molecule comprising a first and a
15 second portion joined together covalently, said first portion comprising a receptor-binding portion of the variant cytokine of claim 1 and said second portion comprising a molecule having enzymatic activity.
4. The hybrid molecule of claim 3 wherein said
20 molecule decreases cell viability.
5. The hybrid molecule of claim 4 wherein said second portion comprises a cytotoxin.
6. The hybrid molecule of claim 5 wherein said cytotoxin is a fragment of a peptide toxin which is
25 enzymatically active but which does not possess generalized eukaryotic receptor binding activity.
7. The hybrid molecule of claim 6 wherein said fragment of a peptide toxin comprises fragment A of

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diphtheria toxin and enough of fragment B of diphtheria toxin to form a pore in a cell membrane.

8. The variant of claim 1 wherein said naturally occurring cytokine is an interleukin.

5 9. The variant of claim 8 wherein said interleukin is IL-4.

10. The variant of claim 9 wherein said IL-4 is human IL-4.

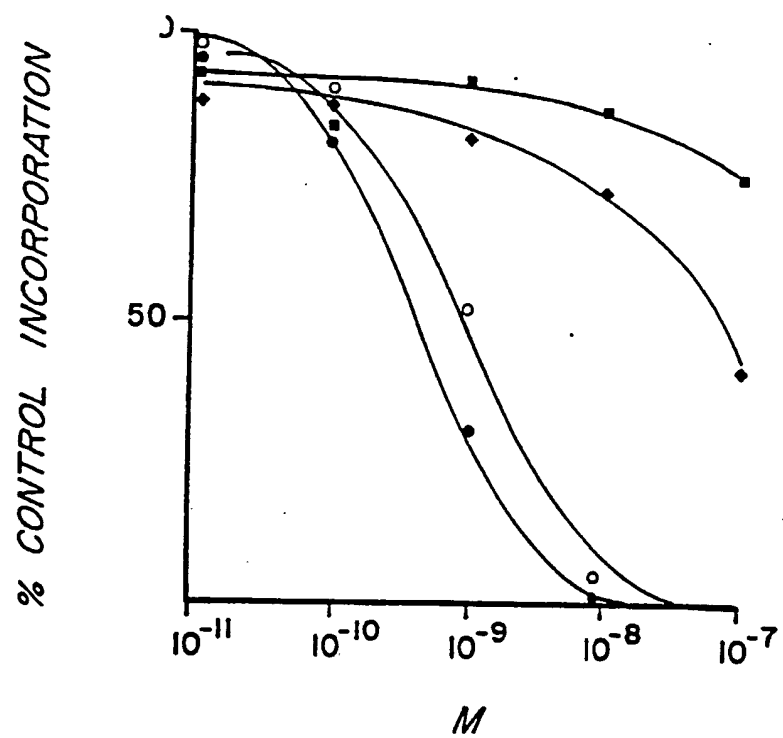


Fig. 1

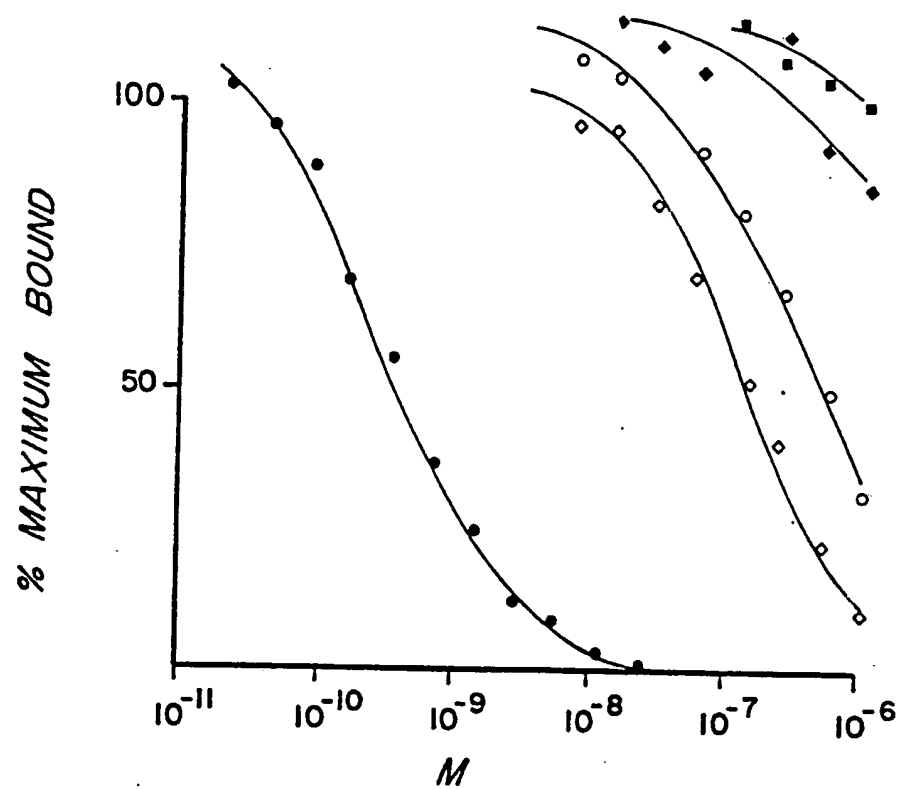


Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/03613

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12N 13/00, 9/00; A61K 37/36

US CL : 530/351; 514/2, 8

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/351; 514/2, 8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Protein Engineering, Volume 5, Number 3, issued April 1992, F. Lakkis et al., "Phe496 and Leu497 are essential for receptor binding and cytotoxic action of the murine interleukin-4 receptor targeted fusion toxin DAB389-mIL4", pages 241-248, especially the abstract, Tables I and II, and Figure 1.	3-7
A	Immunology Today, Volume 11, Number 10, issued October 1990, J. F. Bazan, "Haemopoietic receptors and helical cytokines", pages 350-354.	1-10

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

Special categories of cited documents:	
A document defining the general state of the art which is not considered to be part of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
E earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 July 1993

Date of mailing of the international search report

14 JUL 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

DAVID L. FITZGERALD

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/03613

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Proteins: Structure, Function, and Genetics, Volume 12, issued January 1992, K. Kaushansky, "Structure-Function Relationships of the Hematopoietic Growth Factors", pages 1-9.	1-10
A	Proteins: Structure, Function, and Genetics, Volume 11, issued 1991, B. M. Curtis et al., "Experimental and Theoretical Studies of the Three-Dimensional Structure of Human Interleukin-4", pages 111-119.	1-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/03613

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

DATABASES: USPTO-APS; Dialog ("OneSearch" of all Biochem., Biotech. and Patent
databases)

SEARCH TERMS: (Interleukin/IL)-4; mutein; site-directed; mutagen?